Isolation of a cinnamic acid-metabolizing *Clostridium glycolicum* strain from oil mill wastewaters and emendation of the species description

¹ Laboratoire de Microbiologie IRD, IFR-BAIM, Universités de Provence et de la Méditerranée, ESIL case 925, 163 Avenue de Luminy, 13288 Marseille Cedex 9, France

Mohamed Chamkha,¹ Marc Labat,¹ Bharat K. C. Patel² and Jean-Louis Garcia¹

Author for correspondence: Jean-Louis Garcia. Tel: +33 4 9182 8572. Fax: +33 4 9182 8570.
e-mail: garcia@esil.univ-mrs.fr

² Microbial Research and Discovery Unit, School of Biomolecular and Biomedical Sciences, Faculty of Science, Griffith University, Brisbane, Queensland 4111, Australia

A strictly anaerobic, Gram-positive, motile, sporulated bacterium, designated strain CIN5, was isolated from olive mill wastewaters after enrichment on cinnamic acid. The rod-shaped cells were slightly curved (0.4–1.1 × 2.0–15 µm) and occurred singly or in pairs. Strain CIN5 utilized a limited number of carbohydrates (glucose, fructose, maltose, sorbitol), grew optimally at 37 °C and at pH 7.3–7.5 and had a DNA G+C content of 29.1±0.3 mol %. Strain CIN5 was very closely related to *Clostridium glycolicum* DSM 1288^T. Both strain CIN5 and the type strain of *C. glycolicum* transformed cinnamic acid to hydrocinnamic acid and a wide range of other cinnamic acid derivatives, including *o*-, *m*- and *p*-coumaric, *o*-, *m*- and *p*-methoxycinnamic, *p*-methylcinnamic, caffeic, ferulic and isoferulic acids, to their corresponding 3-phenylpropionic acids by reducing the double bond of the side chain. Glucose supplementation increased the rate of conversion markedly. The emendation of the description of *C. glycolicum* is proposed to include these new characteristics.

Keywords: *Clostridium glycolicum*, aromatic compounds, metabolism, emendation, oil mill wastewater

INTRODUCTION

Olive mill wastewater (OMW) is a major environmental problem in the Mediterranean region, where more than 30 million m³ of this residue are produced annually. The press processing of 1 ton of olives produces 0.5–0.8 m³ of OMW (Boari *et al.*, 1984). The OMW has a dark brown colour, an unpleasant odour and a variable and heterogeneous chemical composition that depends on the olive species, the stage of ripening, the oil-extraction process and the climatic conditions. Chemically, OMW contains sugars, tannins, polyphenols, polyalcohols, pectins, lipids and a wide variety of simple aromatic compounds resulting from olive cell-wall degradation during the oil-extraction process. The main simple phenolic compounds in OMW include tyrosol, hydroxytyrosol and syringic,

p-hydroxyphenylacetic, vanillic, veratric, protocatechuic, caffeic, *p*-coumaric and cinnamic acids (Balice & Cera, 1984; Hamdi, 1993; Labat *et al.*, 2000). The maximum BOD (biological oxygen demand) and COD (chemical oxygen demand) reach 100 and 220 kg m⁻³, respectively (Balice *et al.*, 1982). The polyphenols of OMW can inhibit the growth of some bacteria and Hep2 human cells (Capasso *et al.*, 1995).

Catabolism of the main cinnamic compounds derived from the degradation of lignin and other aromatic constituents of plants (*p*-coumaric, caffeic, ferulic and cinnamic acids) is an important step of the carbon cycle by which natural aromatics and many industrial pollutants are degraded (Andreoni & Bestetti, 1986). The final fate of these compounds is to reach the soil (Subba Rao *et al.*, 1971). Strains of bacteria, yeast and fungi have been shown to metabolize hydroxycinnamic acids and to be resistant to the inhibitory effects of these compounds (Edlin *et al.*, 1994). Most often, free cinnamic acid derivatives are metabolized by different micro-organisms into 4-vinyl derivatives and then

Abbreviation: OMW, olive mill wastewater.

The GenBank accession number for the 16S rRNA gene sequence of strain CIN5 is AY007244.

reduced into 4-ethyl derivatives; alternatively, these phenolic acids can be reduced first into their substituted phenylpropionic acids and then decarboxylated to generate 4-ethyl derivatives. These volatile phenols are potential additives for improving the aroma of wine and other fermented foods and beverages (Cavin *et al.*, 1997). Reduction of the double bond in the side chain of some cinnamic acid derivatives was reported in the rat intestinal microflora (Scheline, 1968), anaerobic microbial consortia (Nali *et al.*, 1985; Angeles de la Torre & Gomez-Alarcon, 1991), methanogenic consortia (Grbic-Galic & Young, 1985), *Pseudomonas cepacia* (Andreoni *et al.*, 1984), *Pseudomonas stutzeri* and *Pseudomonas putida* (Andreoni & Bestetti, 1986), *Wolinella succinogenes* (Ohmiya *et al.*, 1986) and *Corynebacterium glutamicum* (Labuda *et al.*, 1993).

We report in this paper the isolation and the characterization of a strain of *Clostridium glycolicum* from OMW that reduced a wide range of cinnamic acids. We propose to emend the description of the species *C. glycolicum*.

METHODS

Source of strains. Liquid samples of OMW were collected from the lagoon of an oil olive factory near Marseilles, France, and inoculated anaerobically into basal medium containing 5 mM cinnamic acid as described below. Strain CIN5 was isolated from these enrichment cultures. *C. glycolicum* DSM 1288^T was used as a reference culture and was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The reference culture was routinely grown using glucose-containing basal medium.

Culture media. The anaerobic techniques of Hungate (Hungate, 1969; Macy *et al.*, 1972; Miller & Wolin, 1974) were used throughout this work. The basal medium contained (l⁻¹): 0.4 g NH₄Cl, 0.5 g KH₂PO₄, 0.4 g NaCl, 0.33 g MgCl₂·6H₂O, 0.05 g CaCl₂·2H₂O, 0.25 g cysteine/HCl, 2 g yeast extract (Difco), 1 ml trace element mineral solution (Widdel & Pfennig, 1981) and 1 mg resazurin. The pH was adjusted to 7 with 10 M KOH and the medium was then boiled under a stream of O₂-free N₂ gas and cooled to room temperature. Five ml aliquots were dispensed into Hungate tubes, degassed under N₂/CO₂ (80:20%) and subsequently sterilized by autoclaving at 110 °C for 45 min. Prior to inoculation, 0.05 ml 10% (w/v) NaHCO₃ and 0.05 ml 5% (w/v) Na₂S·9H₂O were injected from sterile stock solutions. Substrates were injected from concentrated anaerobic sterile stock solutions to obtain the desired final concentration.

Enrichment and isolation procedure. Samples (0.5 ml) were inoculated into 5 ml basal medium containing 5 mM cinnamic acid and then incubated at 37 °C. The enrichment was subcultured several times under the same conditions prior to isolation. For isolation, the culture was serially diluted tenfold and single well-isolated colonies that developed in roll tubes (basal medium containing 5 mM cinnamic acid and 1.6% agar) were picked. This procedure was repeated several times until only one type of colony was observed. Strain CIN5 was maintained in basal medium containing 5 mM cinnamic acid.

Morphology and sporulation test. Light and electron microscopy were performed as described by Fardeau *et al.* (1997). For testing heat resistance, cells grown in basal medium containing glucose were exposed to temperatures of 80, 90 and 100 °C for 10 min. The cultures were cooled quickly to ambient temperature and inoculated into fresh glucose-containing medium and growth was recorded after 24 h of incubation at 37 °C. Conditions for sporulation that were tested included growth in the presence of glucose or cinnamic acid or with no added carbon source.

Growth parameters. For all experiments, basal medium containing 0.2% yeast extract and 10 mM glucose was used. The pH of the pre-reduced anaerobic medium was adjusted with 5% NaHCO₃, 5% Na₂CO₃ or 0.1 M HCl to obtain a range of initial pH values. Different amounts of NaCl were weighed directly in Hungate tubes prior to dispensing 5 ml medium to obtain the desired NaCl concentration.

Electron acceptors. Sulfate, thiosulfate, sulfite, nitrate and fumarate (20 mM) and elemental sulfur (2%, w/v) were tested as electron acceptors in basal medium containing 10 mM glucose.

Substrate utilization. Experiments were performed in duplicate with an inoculum subcultured at least once under the same test conditions. The substrates tested for utilization were injected from pre-sterilized and concentrated stock solutions into Hungate tubes that contained 5 ml pre-sterilized basal medium. The following substrates were tested: 20 mM carbohydrates, 20 mM organic acids, 20 mM alcohols, 20 mM betaine, 10 g peptides l⁻¹ and 5 mM aromatic compounds. The concentrated stock solutions were prepared, neutralized if necessary, rendered anaerobic by gassing with O₂-free N₂ and sterilized by filtration (pore size 0.2 µm; Millipore). Aromatic compounds were tested with or without 5 mM glucose. Autotrophic growth was tested using H₂/CO₂ (20:80%) at a final pressure of 2 bar (200 kPa). An increase in OD₅₈₀ in tubes containing added substrates, compared with control tubes lacking a substrate, was considered to be positive growth.

Analytical techniques. Bacterial growth was monitored by measuring OD₅₈₀ directly from anaerobic Hungate tubes inserted into the cuvette holder of a spectrophotometer (Shimadzu UV 160A). Aromatic compounds were measured by HPLC by using a chromatograph (Consta Metric 200; LDC-Analytical) equipped with a C₁₈ Symmetry 5 µm-particle-size column, 250 mm long, 4.6 mm i.d. (Waters Chromatography). The column temperature was maintained at 35 °C. An isocratic mobile phase of 30:69.5:0.5 (by vol) acetonitrile/distilled water/acetic acid was used at a flow rate of 0.6 ml min⁻¹. The volume of the injection loop was 25 µl. Aromatic compounds were quantified at 240 nm with a Shimadzu SPD-6A UV detector connected to a CR-6A Shimadzu integrator. Carbohydrates, volatile fatty acids and alcohols were measured by HPLC (Spectra Series 100 model; Thermo Separation Products) equipped with an Aminex HPX-87X 300 mm long, 7.8 mm i.d. column (Bio-Rad) connected to a differential refractometer (RID-6A, Shimadzu). Analysis was performed using a CR-6A Shimadzu integrator. The mobile phase was 2.5 mM H₂SO₄ at a flow rate of 0.5 ml min⁻¹ and the column temperature was 35 °C. The volume of the injection loop was 20 µl. H₂ and CO₂ were measured as described by Fardeau *et al.* (1993).

Determination of the G+C content and DNA-DNA hybridization. The G+C content of DNA was determined

by the DSMZ. The DNA was isolated and purified by chromatography on hydroxyapatite (Cashion *et al.*, 1977) and the G+C content was determined by using HPLC as described by Mesbah *et al.* (1989). Non-methylated lambda DNA (Sigma) was used as the standard. DNA–DNA hybridization was performed at the DSMZ as described by De Ley *et al.* (1970), with the modification described by Escara & Hutton (1980) and Huß *et al.* (1983), using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke & Bahnweg (1986) and Jahnke (1992).

16S rRNA sequence studies. DNA was extracted as described by Redburn & Patel (1993) and Andrews & Patel (1996). The universal primers Fd1 and Rd1 were used to obtain a PCR product of approximately 1.5 kb corresponding to base positions 8 to 1542 based on *Escherichia coli* numbering of the 16S rDNA (Winker & Woese, 1991). The amplification of 16S rRNA, sequencing of PCR products and alignment of sequences were performed as described by Baena *et al.* (1999). Pairwise evolutionary distances based on 1380 unambiguous nucleotides were computed using the programs DNADIST (Jukes & Cantor, 1969) and NEIGHBOR that form part of the PHYLIP suite of programs (Felsenstein, 1993). TREECON was used extensively for bootstrap analysis (Van de Peer & De Wachter, 1993).

RESULTS AND DISCUSSION

Enrichment and isolation

Growth and acetate production were observed on enrichment cultures in medium containing 5 mM cinnamic acid within 2 weeks of incubation at 37 °C. After several transfers in liquid medium, a stable microbial population developed that degraded cinnamic acid. Several isolates were obtained using the roll-tube method (Hungate, 1969) and one of these cultures, designated strain CIN5, was studied further. Isolation of strain CIN5 from OMW on cinnamic acid is not surprising, as the biotope contains a wide range of aromatic compounds resulting from olive cell-wall degradation during oil extraction (Balice & Cera, 1984; Hamdi, 1993; Labat *et al.*, 2000). Recently, other strictly anaerobic bacteria were isolated from a methanogenic pilot-scale digester fed with OMW. Two of these, *Eubacterium aggregans* (Mechichi *et al.*, 1998) and *Clostridium methoxybenzovorans* (Mechichi *et al.*, 1999a), demethylate some methoxylated aromatic compounds without ring cleavage. Another anaerobe found in this digester, *Sporobacterium olearium*, utilizes a range of aromatic compounds, methanol and crotonate as carbon and energy sources. Demethoxylation is the initial key reaction for the subsequent degradation of methoxylated compounds (Mechichi *et al.*, 1999b).

Morphology and physiology

Cells of strain CIN5 were Gram-positive, straight to slightly curved rods, 0.4–1.1 × 2.0–15 µm and motile and occurred singly or in pairs. Sporulated cells were

not abundant in late exponential growth phase. However, sporulation occurred readily after incubation of culture for 10 min at 80 °C. Spores were oval, usually subterminal and non-swelling. Positive growth was obtained in fresh medium after inoculation of cultures that had been incubated for 10 min at 80 °C, but not at 90 °C or 100 °C, indicating resistance to heat.

Strain CIN5 is a mesophilic and strictly anaerobic bacterium. The temperature range for growth was 20–42 °C, with optimal growth occurring at 37 °C. No growth occurred at 10 or 45 °C. The initial pH range for growth was pH 6.0–9.4 with an optimum at pH 7.3–7.5. The NaCl concentration range for growth was 0–50 g l⁻¹. Growth was inhibited markedly above 40 g NaCl l⁻¹. Yeast extract was required for growth and could be used as the sole carbon and energy source. Strain CIN5 fermented ethylene glycol, propylene glycol and glycerol, a limited number of carbohydrates including glucose, fructose, maltose and sorbitol, the organic acid succinate and peptides including yeast extract, Casamino acids, Biotryptcase and peptone. Isolate CIN5 did not utilize arabinol, cellobiose, galactose, lactose, mannitol, mannose, melibiose, *myo*-inositol, raffinose, ribose, sorbose, trehalose, acetate, adipate, butyrate, crotonate, formate, isobutyrate, lactate, malate, propionate, valerate, ethanol, 2-propanol, methanol, propanol, betaine, gelatin or H₂/CO₂. Ethylene glycol was fermented into acetate and ethanol, propylene glycol was fermented into propionate, propanol and small amounts of acetate and ethanol, glycerol was fermented into ethanol, acetate, CO₂ and H₂, glucose was fermented into ethanol, formate, acetate, CO₂ and H₂ and succinate was fermented into propionate and CO₂. Acetate, isovalerate, ethanol, H₂ and CO₂ were produced from the utilization of yeast extract, Casamino acids and Biotryptcase. Acetate, isovalerate and CO₂ were produced from peptone utilization. Sulfate, thiosulfate, sulfite, elemental sulfur, nitrate and fumarate could not be used as electron acceptors.

G+C content, 16S rRNA sequence analysis and DNA–DNA relatedness

The DNA G+C content of strain CIN5 was 29.1 ± 0.3 mol %. Using twelve primers, we determined an almost complete sequence consisting of 1493 bases of the 16S rRNA gene of strain CIN5 (corresponding to *E. coli* positions 15–1539; Winker & Woese, 1991). Phylogenetic analysis revealed that strain CIN5 was a member of the low-G+C-content Gram-positive branch and grouped with members of cluster XI (Collins *et al.*, 1994). Strain CIN5 was found to be closely related to *C. glycolicum* (Gaston & Stadtman, 1963), with only four nucleotide differences between the two 16S rRNA sequences. The level of DNA–DNA relatedness between strain CIN5 and *C. glycolicum* DSM 1288^T was 79 %. The DNA G+C content of strain CIN5 is the same that of *C. glycolicum* (Johnson & Francis, 1975). Phenotypically, both strains are

straight to slightly curved rods, occurring singly or in pairs, motile and spore-formers. They grow at mesophilic temperatures, ferment ethylene glycol, propylene glycol, a limited number of carbohydrates and some peptides and are unable to hydrolyse gelatin (Cato *et al.*, 1986). The phylogenetic, genotypic and phenotypic characteristics of isolate CIN5 indicate that it is a member of the genus *Clostridium* and a strain of *C. glycolicum*.

C. glycolicum has been isolated from soil, mud, snake venom, bovine intestine, human faeces and clinical specimens including wounds, abscesses and peritoneal fluid (Drasar *et al.*, 1976; Finegold *et al.*, 1983). The isolation of strain CIN5 from OMW extends the known habitat for *C. glycolicum*.

Metabolism of aromatic compounds

Strain CIN5 metabolized various cinnamic acids to their corresponding 3-phenylpropionic (hydrocinnamic) acid derivatives. After 2 d of anaerobic incubation at 37 °C, isolate CIN5 converted cinnamic acid to 3-phenylpropionic acid, *o*-coumaric acid to 3-(2-hydroxyphenyl) propionic acid, *m*-coumaric acid to 3-(3-hydroxyphenyl) propionic acid, *p*-coumaric acid to 3-(4-hydroxyphenyl) propionic acid, *o*-methoxycinnamic acid to 3-(2-methoxyphenyl) propionic acid, *m*-methoxycinnamic acid to 3-(3-methoxyphenyl) propionic acid, *p*-methoxycinnamic acid to 3-(4-methoxyphenyl) propionic acid, *p*-methylcinnamic acid to 3-(4-methylphenyl) propionic acid and isoferulic acid to 3-(3-hydroxy-4-methoxyphenyl) propionic acid, with or without 5 mM glucose. Caffeic acid and ferulic acid were metabolized by strain CIN5 to 3-(3,4-dihydroxyphenyl) propionic acid at 52% and 3-(4-hydroxy-3-methoxyphenyl) propionic acid at 57%, respectively, after 2 d of incubation. However, with glucose supplementation, the conversion of caffeic and ferulic acids was complete after 2 d. Transformation was complete, regardless of the presence of glucose in the medium, after 4 d of incubation.

The amount of phenylpropionic acid derivatives produced by strain CIN5 equalled that of cinnamic acids consumed. The ratio of phenylpropionic acids to their corresponding cinnamic acids in the medium increased during the course of the experiment until substrate utilization was complete. By contrast, strain CIN5 did not reduce 3,4,5-trimethoxycinnamic acid; substitution of both of the *meta* positions and the *para* position (3,4,5) with methoxyl groups on the aromatic ring inhibited the biotransformation. Cinnamyl alcohol, cinnamaldehyde, styrene, oestragole (4-allyl-anisole), eugenol (4-allyl-2-methoxyphenol) and phenylpropionic acids [3-phenylpropionic acid (hydrocinnamic acid) and 3,4-dihydroxyphenylpropionic acid (hydrocaffeic acid)] were not metabolized by strain CIN5, indicating that the composition of the side chain determined the substrate specificity; an acrylic acid side chain was required by *C. glycolicum* for the biotransformation of aromatic compounds.

Other aromatic compounds tested at 5 mM in basal medium, with or without 5 mM glucose, were not used by strain CIN5. They included benzoate, hydroxylated benzoic acids [4-hydroxybenzoate, 2,4-, 2,6-, 3,4- and 3,5-dihydroxybenzoates, 2,4,6-trihydroxybenzoate and 3,4,5-trihydroxybenzoate (gallate)], methoxylated benzoic acids (2,4-, 2,6-, 3,4- and 3,5-dimethoxybenzoates and 3,4,5-trimethoxybenzoate), mixed hydroxylated/methoxylated benzoic acids [4-hydroxy-3-methoxybenzoate (vanillate) and 3-hydroxy-4-methoxybenzoate (isovanillate)], 3,4-dimethylbenzoic acid, gallic acid methyl ester, *p*-hydroxyphenylethanol (1,4-tyrosol) and 3,4,5-trihydroxybenzene (pyrogallol). No ring cleavage was observed with any of the aromatic compounds tested, even after 2 weeks of incubation.

The metabolism of cinnamic compounds by the type strain of *C. glycolicum* was similar to that observed with strain CIN5. The metabolism of various cinnamic acids by isolate CIN5 and the type strain of *C. glycolicum* involved the reduction of the double bond in the side chain. Corresponding phenylpropionic acids were always the end-products, without further degradation. With all aromatic substrates used by both strains, adding glucose enhanced growth and accelerated this conversion markedly. The presence of cinnamic acids did not result in higher growth yields, so cinnamic acids cannot be used as the carbon and energy source. Cinnamic acids function in both of these strains as terminal electron acceptors in the oxidation of H₂. Hydrogen produced from yeast extract or glucose utilization can be used by strain CIN5 or the type strain of *C. glycolicum* to reduce the double bond in the side chain. Many micro-organisms cannot degrade aromatic compounds completely, but catalyse oxygen-independent biotransformations without ring cleavage. The effect of reductive anaerobic biotransformations of aromatic substrates is to dispose of redox equivalents generated in oxidative reactions. The known reductive biotransformation reactions are either involved in anaerobic respiratory chains or are part of fermentative pathways (Heider & Fuchs, 1997). The processes are exergonic and in some cases are even associated with energy conservation.

Our results indicate that both the type strain of *C. glycolicum* and strain CIN5 transform 11 cinnamic acids and, hence, we propose to emend the description of *C. glycolicum* to include these new traits.

Emended description of *Clostridium glycolicum* (Gaston and Stadtman 1963)

Clostridium glycolicum (gly.co'li.cum. L. adj. suff. *-icus* related to, belonging to; N.L. neut. adj. *glycolicum* referring to the ability to ferment ethylene glycol).

Cells are strictly anaerobic, Gram-positive, straight to slightly curved rods, motile and peritrichous, 0.3–1.3 × 1.8–15.4 µm, occurring singly or in pairs. Spores are oval and usually subterminal, occasionally terminal, often occurring as free spores and not swelling.

Surface colonies are 1–4 mm, circular to irregular, raised to convex, translucent to semi-opaque, greyish white, shiny and smooth with a granular, mottled or mosaic internal structure and entire, scalloped or erose margins. The optimum temperature for growth is 30–37 °C at pH 7.3–7.6. Most strains grow moderately well at 20 °C, poorly if at all at 45 °C. Growth is inhibited by 6.5% NaCl, 20% bile and pH 9.5. Yeast extract is required for growth and can be used as the sole carbon and energy source. Ferments ethylene glycol to acetate and ethanol and propylene glycol to propionate, propanol and small amounts of acetate and ethanol. Sugars fermented are glucose, fructose, sorbitol, xylose, maltose and dulcitol, with acid and gas formation. Succinate is fermented into propionate and CO₂, glycerol is fermented into acetate, ethanol, H₂ and CO₂, yeast extract, Casamino acids and Biotryptase are fermented into acetate, isovalerate, ethanol, H₂ and CO₂ and peptone is fermented into acetate, isovalerate and CO₂. Substrates not fermented include amygdalin, arabinose, cellobiose, galactose, glycogen, inulin, lactose, mannitol, mannose, melzitose, melibiose, raffinose, rhamnose, ribose, salicin, sucrose and trehalose. Degrades uracil to β -alanine, ammonia and CO₂. Threonine is converted to propionate. Products in PYG broth are acetic, isovaleric and isobutyric acids and ethyl, propyl, isobutyl and isoamyl alcohols; propionic, formic, lactic and succinic acids may also be detected. Abundant H₂ is produced. Lecithinase, lipase, indole and caproic and butyric acids are not produced. Starch and gelatin are not hydrolysed. Sulfate, thiosulfate, sulfite, elemental sulfur, nitrate and fumarate are not reduced and lactate is not utilized. Reduces the double bond of the side chain of a wide range of cinnamic acids: cinnamic, *o*-, *m*- and *p*-coumaric, *o*-, *m*- and *p*-methoxycinnamic, *p*-methylcinnamic, caffeic, ferulic and isoferulic acids. The end-products of metabolism of cinnamic acids are the corresponding phenylpropionic acids: 3-phenyl, 3-(2-hydroxyphenyl), 3-(3-hydroxyphenyl), 3-(4-hydroxyphenyl), 3-(2-methoxyphenyl), 3-(3-methoxyphenyl), 3-(4-methoxyphenyl), 3-(4-methylphenyl), 3-(3,4-dihydroxyphenyl), 3-(4-hydroxy-3-methoxyphenyl) and 3-(3-hydroxy-4-methoxyphenyl) propionic acids. Glucose supplementation increases the rate of conversion markedly. The genomic DNA G + C content is 29 mol%. Member of cluster XI of the low-G + C-content Gram-positive branch. The habitat includes soil, mud, oil mill wastewater, snake venom, bovine intestine, human faeces and clinical specimens including wounds, abscesses and peritoneal fluid.

The type strain is ATCC 14880^T (= NCIB 10632^T = DSM 1288^T). Strain CIN5 (= DSM 13561 = CIP 106672) is a reference strain.

ACKNOWLEDGEMENTS

Financial assistance to M.C. from the Tunisian Minister for Superior Teaching and the French Research Institute for Development and to B.K.C.P. from the Australian Re-

search Council is gratefully acknowledged. We are indebted to P. A. Roger for improving the manuscript.

REFERENCES

- Andreoni, V. & Bestetti, G. (1986). Comparative analysis of different *Pseudomonas* strains that degrade cinnamic acid. *Appl Environ Microbiol* **52**, 930–934.
- Andreoni, V., Galli, E. & Galliani, G. (1984). Metabolism of ferulic acid by a facultatively anaerobic strain of *Pseudomonas cepacia*. *Syst Appl Microbiol* **5**, 299–304.
- Andrews, K. T. & Patel, B. K. C. (1996). *Fervidobacterium gondwanense* sp. nov., a new thermophilic anaerobic bacterium isolated from nonvolcanically heated geothermal waters of the Great Artesian Basin of Australia. *Int J Syst Bacteriol* **46**, 265–269.
- Angeles de la Torre, M. & Gomez-Alarcon, G. (1991). Ferulic acid anaerobic degradation by marsh sediment microorganisms. *Microbios* **68**, 119–127.
- Baena, S., Fardeau, M.-L., Ollivier, B., Labat, M., Thomas, P., Garcia, J.-L. & Patel, B. K. C. (1999). *Aminomonas paucivorans* gen. nov., sp. nov., a mesophilic, anaerobic, amino-acid-utilizing bacterium. *Int J Syst Bacteriol* **49**, 975–982.
- Balice, V. & Cera, O. (1984). Acidic phenolic fraction of the juice of olives determined by gas chromatographic method. *Grasas Aceites* **25**, 178–180.
- Balice, V., Boari, G., Cera, O. & Abbaticchio, P. (1982). Indagine analitica sulle acque di vegetazione. Nota 1. *Inquinamento* **7**, 49–53.
- Boari, G., Brunetti, A., Passino, R. & Rozzi, A. (1984). Anaerobic digestion of olive oil mill wastewaters. *Agric Wastes* **10**, 161–175.
- Capasso, R., Evidente, A., Schivo, L., Orru, G., Marcialis, M. A. & Cristinzio, G. (1995). Antibacterial polyphenols from olive oil mill waste waters. *J Appl Bacteriol* **79**, 393–398.
- Cashion, P., Holder-Franklin, M. A., McCully, J. & Franklin, M. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461–466.
- Cato, E. P., George, W. L. & Finegold, S. M. (1986). Genus *Clostridium* Prazmowski 1880. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1141–1200. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Cavin, J.-F., Barthelmebs, L. & Diviès, C. (1997). Molecular characterization of an inducible *p*-coumaric acid decarboxylase from *Lactobacillus plantarum*: gene cloning, transcriptional analysis, overexpression in *Escherichia coli*, purification, and characterization. *Appl Environ Microbiol* **63**, 1939–1944.
- Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* **44**, 812–826.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Drasar, B. S., Goddard, P., Heaton, S., Peach, S. & West, B. (1976). Clostridia isolated from faeces. *J Med Microbiol* **9**, 63–71.
- Edlin, D. A. N., Narbad, A., Dickinson, J. R. & Lloyd, D. (1994). The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiol Lett* **125**, 311–316.
- Escara, J. F. & Hutton, J. R. (1980). Thermal stability and

renaturation of DNA in dimethyl sulfoxide solutions: acceleration of the renaturation rate. *Biopolymers* **19**, 1315–1327.

Fardeau, M.-L., Cayol, J.-L., Magot, M. & Ollivier, B. (1993). H₂ oxidation in the presence of thiosulfate, by a *Thermoanaerobacter* strain isolated from an oil-producing well. *FEMS Microbiol Lett* **113**, 327–332.

Fardeau, M.-L., Ollivier, B., Patel, B. K. C., Magot, M., Thomas, P., Rimbault, A., Rocchiccioli, F. & Garcia, J.-L. (1997). *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. *Int J Syst Bacteriol* **47**, 1013–1019.

Felsenstein, J. (1993). PHYLIP (Phylogenetic Inference Package) version 3.51c. Distributed by the author, Department of Genetics, University of Washington, Seattle, WA, USA.

Finegold, S. M., Sutter, V. L. & Mathisen, G. E. (1983). Normal indigenous intestinal flora. In *Human Intestinal Microflora in Health and Disease*, pp. 3–31. Edited by D. J. Hentges. New York: Academic Press.

Gaston, L. W. & Stadtman, E. R. (1963). Fermentation of ethylene glycol by *Clostridium glycolicum*, sp. n. *J Bacteriol* **85**, 356–362.

Grbic-Galic, D. & Young, L. Y. (1985). Methane fermentation of ferulate and benzoate: anaerobic degradation pathways. *Appl Environ Microbiol* **50**, 292–297.

Hamdi, H. (1993). Future prospects and constraints of olive mill wastewaters use and treatment: a review. *Bioprocess Eng* **8**, 209–214.

Heider, J. & Fuchs, G. (1997). Microbial anaerobic aromatic metabolism. *Anaerobe* **3**, 1–22.

Hungate, R. E. (1969). A roll-tube method for the cultivation of strict anaerobes. *Methods Microbiol* **136**, 194–198.

HuB, V. A. R., Festel, H. & Schleifer, K. H. (1983). Studies on the spectrometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.

Jahnke, K.-D. (1992). Basic computer program for evaluation of spectroscopic DNA renaturation data from GILFORD System 2600 spectrometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15**, 61–73.

Jahnke, K.-D. & Bahnweg, G. (1986). Assessing natural relationships in the basidiomycetes by DNA analysis. *Trans Br Mycol Soc* **87**, 175–191.

Johnson, J. L. & Francis, B. S. (1975). Taxonomy of the clostridia: ribosomal ribonucleic acid homologies among the species. *J Gen Microbiol* **88**, 229–244.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Labat, M., Augur, C., Perraud-Gaime, I., Roussos, S. & Sayadi, S. (2000). Biotechnological potentialities of polyphenolic compounds of coffee and comparison with olive. In *Coffee Biotechnology and Quality*, pp. 517–531. Edited by T. Sera, C. R. Soccol, A. Pandey & S. Roussos. Dordrecht: Kluwer.

Labuda, I. M., Keon, K. A. & Goers, S. K. (1993). Microbial bioconversion process for the production of vanillin. In

Progress in Flavor Precursor Studies. Analysis–Generation–Biotechnology, pp. 477–482. Edited by P. Schreier & P. Winterhalter. Carol Stream, IL: Allured.

Macy, J. M., Snellen, J. E. & Hungate, R. E. (1972). Use of syringe methods for anaerobiosis. *Am J Clin Nutr* **25**, 1318–1323.

Mechichi, T., Labat, M., Woo, T. H. S., Thomas, P., Garcia, J.-L. & Patel, B. K. C. (1998). *Eubacterium aggregans* sp. nov., a new homoacetogenic bacterium from olive mill wastewater treatment digester. *Anaerobe* **4**, 283–291.

Mechichi, T., Labat, M., Patel, B. K. C., Woo, T. H. S., Thomas, P. & Garcia, J.-L. (1999a). *Clostridium methoxybenzovorans* sp. nov., a new aromatic *o*-demethylating homoacetogen from an olive mill wastewater treatment digester. *Int J Syst Bacteriol* **49**, 1201–1209.

Mechichi, T., Labat, M., Garcia, J.-L., Thomas, P. & Patel, B. K. C. (1999b). *Sporobacterium olearium* gen. nov., sp. nov., a new methanethiol-producing bacterium that degrades aromatic compounds, isolated from an olive mill wastewater treatment digester. *Int J Syst Bacteriol* **49**, 1741–1748.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Miller, T. L. & Wolin, M. J. (1974). A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Appl Microbiol* **27**, 985–987.

Nali, M., Rindone, B., Tollari, S., Andreoni, V. & Treccani, V. (1985). Anaerobic microbial conversion of three hydroxycinnamic acids. *Experientia* **41**, 1351–1353.

Ohmiya, K., Takeuchi, M., Chen, W., Shimizu, S. & Kawakami, H. (1986). Anaerobic reduction of ferulic acid to dihydroferulic acid by *Wolinella succinogenes* from cow rumen. *Appl Microbiol Biotechnol* **23**, 274–279.

Redburn, A. C. & Patel, B. K. C. (1993). Phylogenetic analysis of *Desulfotomaculum thermobenzoicum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. *FEMS Microbiol Lett* **113**, 81–86.

Scheline, R. R. (1968). Metabolism of phenolic acids by the rat intestinal microflora. *Acta Pharmacol Toxicol* **26**, 189–205.

Subba Rao, P. V., Nambudiri, A. M. D. & Bhat, J. V. (1971). Microbial degradation of phenylpropanoid compounds. *J Sci Ind Res* **30**, 663–679.

Van de Peer, Y. & De Wachter, R. (1993). TREECON: a software package for the construction and drawing of evolutionary trees. *Comput Appl Biosci* **9**, 177–182.

Widdel, F. & Pfennig, N. (1981). Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. *Arch Microbiol* **129**, 395–400.

Winker, S. & Woese, C. R. (1991). A definition of the domains Archaea, Bacteria and Eucarya in terms of small subunit ribosomal RNA characteristics. *Syst Appl Microbiol* **13**, 161–165.